INOSITOL PHOSPHATE PHOSPHATASES OF MICROBIOLOGICAL ORIGIN. SOME PROPERTIES OF A PARTIALLY PURIFIED BACTERIAL (*PSEUDOMONAS* SP.) PHYTASE

By G. C. J. Irving* and D. J. Cosgrove*

[Manuscript received November 18, 1970]

Abstract

A phytase (E.C. 3.1.3.8) was extracted from a bacterium (*Pseudomonas* sp.) and purified 25-fold by ammonium sulphate fractionation, gel filtration, and ion-exchange cellulose chromatography. Its properties are compared with the published properties of some plant phytases and a bacterial phytase.

The pH optimum at 40°C is 5·5, and at this pH the kinetics, at substrate concentrations above 18·3 μM, are consistent with partially non-competitive substrate inhibition. At lower substrate concentrations the kinetics deviate from this model.

Of a range of potential substrates tested at 0·36 mM, only *p*-nitrophenyl phosphate and inositol hexaphosphates were hydrolysed. The order of the rates of hydrolysis of the inositol hexaphosphates was *myo-* = *neo-* ⩾D-*chiro-* ≈ L-*chiro-* > *scyllo*-inositol hexaphosphate.

The enzyme is inhibited by 1 mM (+)-tartrate and 1 mM EDTA; 1 mM oxalate and citrate are without effect on the activity of the enzyme while the degree of inhibition of the enzyme by fluoride ion appears to be a function of the cube of the fluoride ion concentration.

I. Introduction

The existence of phytase, *myo*-inositol hexaphosphate phosphohydrolase (E.C. 3.1.3.8), has been detected in a variety of organisms (Sloane-Stanley 1961). Most published studies to date have been on plant phytases (Peers 1953; Nagai and Funahashi 1962; Preece and Grav 1962; Gibbins and Norris 1963; Chang 1967); few studies of bacterial phytases have been published.

Greaves, Anderson, and Webley (1967) described the isolation and some of the properties of a phytase from *Aerobacter aerogenes* and Powar and Jagannathan (1967) isolated and examined a phytase from *Bacillus subtilis*. More recently Cosgrove, Irving, and Bromfield (1970) described the isolation, from soil, of a bacterium (*Pseudomonas* sp.) which possessed marked phytase activity. This present paper records the results of some experiments on the properties of a partially purified preparation of the *Pseudomonas* phytase.

* Division of Plant Industry, CSIRO, P.O. Box 109, Canberra City, A.C.T. 2601.

II. Materials and Methods

(a) *Reagents*

The 98% pure dodecasodium salt of *myo*-inositol hexaphosphate was obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. The purity of this reagent was checked by ion-exchange chromatography (Cosgrove 1969). All other reagents were analytical grade quality.

(b) *Culture of Organism*

The organism had previously been cultured (Cosgrove, Irving, and Bromfield 1970) in a medium containing sodium *myo*-inositol hexaphosphate as the sole carbon source. Subsequent work has shown that the bacterium will grow on *myo*-inositol as sole carbon source and still produce phytase. A medium of the following composition was therefore used to culture the bacterium—per 1000 ml of distilled water: *myo*-inositol 2·0 g; K_2HPO_4 7·0 g; KH_2PO_4 2·0 g; $MgSO_4.7H_2O$ 0·10 g; $(NH_4)_2SO_4$ 1·0 g. Portions of sterile culture medium (200 ml) were inoculated with an inoculum (10·0 ml) prepared from an agar slope by suspending the cells in medium (*b*) (Cosgrove 1969). The cultures were shaken for 45 hr at 25°C.

(c) *Preparation of Cell-free Extract*

The cell-free extract was prepared as described by Cosgrove (1969) and the supernatant was stored at −10°C.

(d) *Phosphate Analyses*

Inorganic orthophosphate was determined by a method described elsewhere (Irving and Cosgrove 1970).

(e) *Enzymic Activity*

All activity determinations were made at 40°C in 20 mM succinic acid–sodium hydroxide buffer (pH 5·5). The composition of the assay solution was as follows: 0·36 mM neutral sodium *myo*-inositol hexaphosphate, 0·1 mM disodium EDTA (London, McHugh, and Hudson 1958), 0·005% Triton X-100 (London, McHugh, and Hudson 1958), 20 mM buffer, and a suitable volume of "diluted enzyme". The latter was prepared by diluting the stock solution of partially purified enzyme 1000-fold with 10 mM succinic acid–sodium hydroxide buffer (pH 5·5) containing 0·1 mM disodium EDTA and 0·005% Triton X-100. The assay conditions for individual experiments are set out in the captions to figures and in Table 2. Under these conditions the rate of liberation of inorganic orthophosphate was found to be constant over the period of time specified in the caption.

One unit of activity is defined as that quantity of enzyme which will liberate 1 μmole of inorganic orthophosphate per minute per 5 ml of assay solution.

Due to the chemical stability of *myo*-inositol hexaphosphate (Desjobert and Fleurent 1954) the substrate blanks in experiments with this substrate were indistinguishable from the reagent blank and so were not routinely measured. Substrate blanks were measured routinely with all other substrates.

(f) *Protein Determinations*

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

(g) *Preparation of Partially Purified* Pseudomonas *Phytase*

The culture medium (6 × 200 ml) yielded 18·2 g (wet weight) of cells. The cell-free extract was diluted to 30·0 ml with 50 mM potassium chloride solution and then fractionated at room temperature and at pH 5·5 with solid ammonium sulphate. The material precipitating between 34 and 63% saturation (the "34–63% fraction") contained the bulk of the activity (Table 1). This fraction was dissolved in 11·5 ml of 5 mM succinic acid–sodium hydroxide buffer (pH 5·5) and then stored at −10°C until required.

(h) *Chromatography of the 34–63% Fraction*

The chromatography was carried out at room temperature. All buffer solutions contained 0·1 mM disodium EDTA and 0·005% Triton X-100 and all columns were equilibrated against 5 mM succinic acid–sodium hydroxide buffer (pH 5·5) prior to use.

A 1·0-ml portion of the 34–63% fraction was diluted to 2·0 ml with 5 mM succinic acid–sodium hydroxide buffer (pH 5·5) and after removal of an 0·05-ml aliquot for assay of specific activity (Table 1) the remainder of the solution was de-salted by passage through a Sephadex G15 column (40 by 2·5 cm, void volume 70 ml) using 5 mM succinic acid–sodium hydroxide buffer (pH 5·5) to wash the enzyme from the column. The initial 65·0 ml of effluent was discarded and the following 20·0 ml of effluent, containing the bulk of the activity, was collected.

TABLE 1

PURIFICATION OF *PSEUDOMONAS* PHYTASE

Fraction	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Percentage Recovery
Cell-free preparation	202	66·6	3·03	—
"34–63% fraction"	217	35·2	6·16	107
Sephadex G15	158	9·2	17·2	78
Whatman DE32	29·6	1·0	29·6	14·7
Whatman CM32 (150 mM)	37·6	0·5	75·2	18·6

An aliquot (0·25 ml) of the Sephadex G15 fraction was removed for assay of specific activity (Table 1) and the remainder was run on to a column of Whatman DE32 anion-exchange cellulose (14·0 by 0·9 cm). The activity was washed from the column with 5 mM succinic acid–sodium hydroxide buffer (pH 5·5), collection of the effluent being begun at the same time as the addition of the G15 fraction. The initial 35·0 ml of effluent contained the bulk of the activity washed from the column.

An aliquot (0·5 ml) of the Whatman DE32 fraction was retained for assay of specific activity (Table 1) and the remainder was adsorbed on to a column of Whatman CM32 cation-exchange cellulose (11·5 by 1·2 cm) and eluted using a stepwise gradient of succinic acid–sodium hydroxide buffer (pH 5·5). Portions of buffer (10·0 ml) of increasing concentration (5 mM → 50 mM → 100 mM → 150 mM → 200 mM) were used and 10-ml fractions were collected. Activity

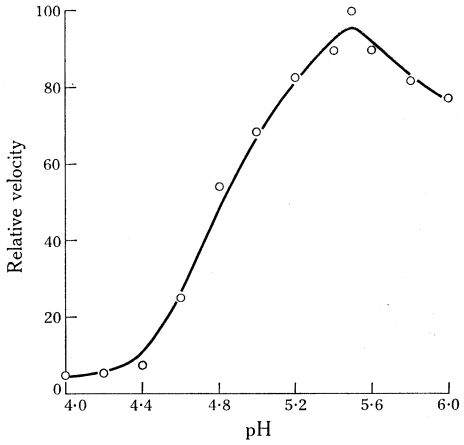

Fig. 1.—Effect of pH on the activity of *Pseudomonas* phytase. For assay conditions see Table 2. Aliquots (1·0 ml) were withdrawn at 0 and 60 min. The relative velocities are expressed as percentages of the initial velocity at pH 5·5. The buffer used was 20 mM succinic acid–sodium hydroxide. Each value is the mean of two determinations.

was eluted by the 150 mM or the 200 mM solution or both. The position of the peak of activity varied from preparation to preparation. An aliquot of the active fraction from the CM32 column was assayed for specific activity (Table 1) and the remainder stored at 5°C until required for use as a stock solution of the enzyme.

III. Results

(*a*) *Partial Purification of* Pseudomonas *Phytase*

Yields and specific activities at the various stages are set out in Table 1.

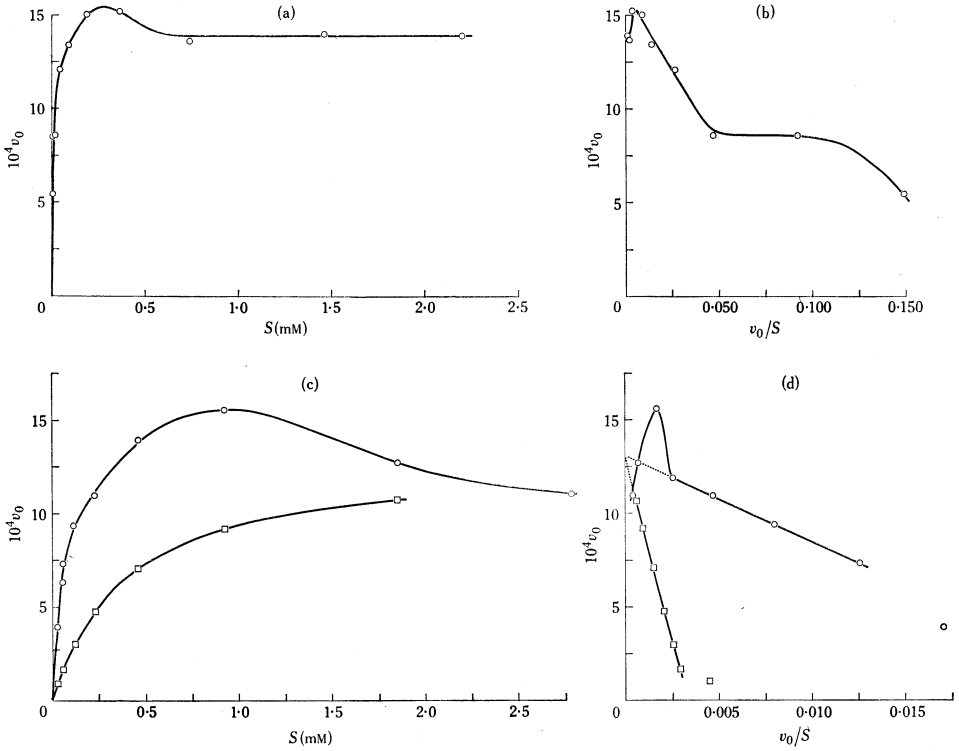

Fig. 2.—(*a*) Activity curve for *Pseudomonas* phytase. The substrate (S) was *myo*-inositol hexaphosphate and the total assay volume was 30·0 ml, containing 1·0 ml of "diluted enzyme" (0·33 μg of protein). Aliquots (5·0 ml) for assay of inorganic orthophosphate were withdrawn at 0, 10, 20, and 30 min after the addition of the diluted enzyme to the assay solutions. For substrate concentrations of 0·0183 mM or greater the initial velocities (v_0 expressed as μmoles inorganic phosphate liberated per minute per 5 ml of assay solution) were estimated from a linear least squares fit to the four values of inorganic orthophosphate obtained. The mean correlation coefficient for these determinations was 0·997 with a standard deviation of 0·003. The assumption of the linearity of the data was therefore valid. In the cases of the two lower concentrations of substrate (0·00916 and 0·00366 mM) the initial velocities were estimated from the values of Pi for the 0–10-min interval, in order to limit the degree of hydrolysis to less than 5%. Each point is the mean of three determinations. (*b*) A Woolf plot of the data from (*a*): $v_{max.} = 1{\cdot}62 \times 10^{-3}$ μmoles inorganic phosphate liberated per minute per 5 ml of assay solution; $K_m = 1{\cdot}63 \times 10^{-5}$M. (*c*) Activity curves for *Pseudomonas* phytase in the presence of 0·5 mM (○) and 5·0 mM (□) magnesium ion. Conditions of assay as for (*a*). (*d*) Woolf plots of the data from (*c*). ○ 0·5 mM magnesium ion: $v_{max.} = 1{\cdot}30 \times 10^{-3}$ μmoles inorganic phosphate liberated per minute per 5 ml of assay solution; $K_m = 4{\cdot}7 \times 10^{-5}$M. □ 5·0 mM magnesium ion: $v_{max.} = 1{\cdot}28 \times 10^{-3}$ μmoles inorganic phosphate liberated per minute per 5 ml assay solution; $K_m = 3{\cdot}9 \times 10^{-3}$M.

(*b*) *Properties of Partially Purified* Pseudomonas *Phytase*

(i) *pH Optimum*

The pH optimum at 40°C in 20 mM succinic acid–sodium hydroxide buffer is 5·5 (Fig. 1).

(ii) *Kinetics*

The substrate–activity curve for the enzyme [Fig. 2(*a*)] is initially concave downward with a peak in the region of 0·3 mM substrate (*myo*-inositol hexaphosphate). At higher substrate concentrations the curve appears to fall to a constant value.

A plot of the activity data using the method proposed by Woolf (quoted by Haldane and Stern 1932) [Fig. 2(*b*)] is linear between values of v_0/S of 0·0469 and 0·0082. At higher values of v_0/S the initial rates are higher than extrapolation would predict while at lower values they are lower.

Magnesium ions appear to inhibit the enzyme competitively and also have the effect of shifting the peak activity to higher substrate concentrations [Figs. 2(*c*), 2(*d*)].

(iii) *Substrate Specificity*

At a substrate concentration of 0·36 mM no activity by the enzyme preparation towards inorganic pyrophosphate, β-glycerophosphate, ADP, or AMP could be detected. The activity against *p*-nitrophenyl phosphate was 14% of that against *myo*-inositol hexaphosphate. The conditions of assay were the same as those for inositol hexaphosphates (Table 2).

TABLE 2

RELATIVE RATES OF HYDROLYSIS OF INOSITOL HEXAPHOSPHATES

Assay Conditions	Substrate	Relative Velocity
The assay solution [see Section II(*e*)] contained 0·2 ml of "diluted enzyme" in a total volume of 5·0 ml. Aliquots (1·0 ml) were taken and assayed for inorganic orthophosphate at 0, 15, and 30 min after the addition of the diluted enzyme. In experiments with mixed substrates the concentration of each substrate was 0·36 mM. Each value is the mean of two determinations	*myo*-	100
	neo-	99
	D-*chiro*-	12
	L-*chiro*-	10
	scyllo-	4
	myo-+*neo*-	119
	myo-+D-*chiro*-	41
	myo-+*scyllo*-	45

The results (Table 2) show that at a substrate concentration of 0·36 mM the enzyme preparation is equally active toward the *myo*- and *neo*- isomers but that it is

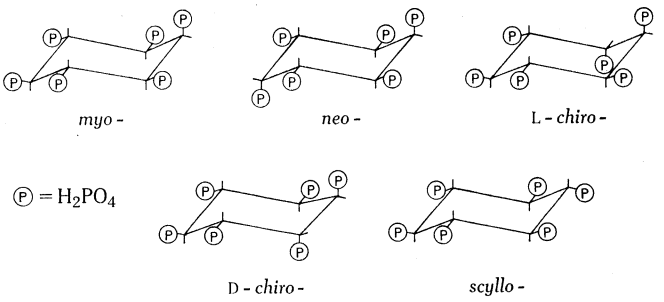

Fig. 3.—The most probable chair conformations of the inositol hexaphosphates discussed in the text.

very much less active toward the D-*chiro*-, L-*chiro*-, and *scyllo*- isomers. The structures of these isomers are illustrated in Figure 3.

The addition of an equimolar quantity of either the D-*chiro*- or the *scyllo*- isomer to an incubation mixture containing *myo*-inositol hexaphosphate as substrate inhibits the enzyme to about the same degree in either case. The similar addition of an equimolar quantity of the *neo*- isomer has a slight activating effect.

(iv) *Effect of Cation-chelating Agents*

The enzyme is not inhibited by either 1·0 mM oxalate or 1·0 mM citrate (relative rates of hydrolysis 101 and 104 respectively in the presence of these chelating agents compared with 100 with no chelating agent present) but it is inhibited, to about the same degree in each case, by 1·0 mM (+)- tartrate and 1·0 mM EDTA (relative rates 87 and 86 respectively). The amount of EDTA contained in the assay solutions for the experiments with oxalate, citrate, and (+)-tartrate was only that in the aliquot of diluted enzyme. The solutions were not made 0·1 mM with respect to EDTA and therefore the concentration of EDTA in these solutions was approximately 4 μM. Other assay conditions are as given in Table 2. Each of the above values is the mean of two determinations.

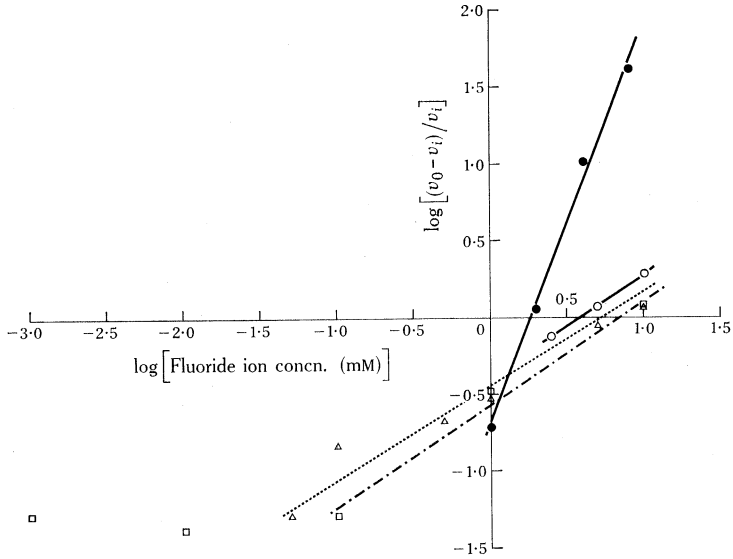

Fig. 4.—A logarithmic plot of fluoride inhibition data for *Pseudomonas* phytase (●), wheat-bran phytase (○), dwarf bean phytase (□), and corn phytase (△). For details see text.

(v) *Effect of Fluoride Ion*

Pseudomonas phytase is inhibited by fluoride ion, as is shown in the following tabulation:

Fluoride ion concn. (mM):	0	1·0	2·0	4·0	8·0
Relative velocity:	100	84	47	9	2·4

Conditions of assay are as given in Table 2 and each value given above is the mean of two determinations.

The results for *Pseudomonas* phytase together with those for wheat-bran phytase (Nagai and Funahashi 1962), dwarf bean phytase (Gibbins and Norris 1963), and corn phytase (Chang 1967) are plotted in Figure 4, using a logarithmic transformation of the equations for fully competitive and fully non-competitive inhibition which were published by Hunter and Downs (1945). These transformations for fully competitive inhibition are:

$$\log[(v_0-v_i)/v_i] = \log[(K_m/K_i)/(S+K_m)]+n\log[F^-], \quad (1)$$

and for fully non-competitive inhibition:

$$\log[(v_0-v_i)/v_i] = \log K_i+n\log[F^-], \quad (2)$$

where v_0 = initial velocity without added flouride, v_i = initial velocity with added fluoride, and $[F^-]$ = total fluoride concentration. The symbols K_m, K_i, and S have their usual meanings and the symbol n, which has been introduced to take into account the fact that the slopes of the plots (Fig. 4) are not unity, may be interpreted as the number of fluorine atoms combined at each active site.

Equations (1) and (2) are equations for straight lines. If the inhibition of these enzymes by fluoride were partially competitive, partially non-competitive, or mixed then plots of log $(v_0-v_i)/v_i$ against log $[F^-]$ would be curved. With the exception of the plot for dwarf bean phytase the plots are linear over the range of data available.

The slope (n) of the plot for *Pseudomonas* phytase is 2·59 while the slopes for wheat-bran phytase, for the linear portion of the dwarf bean phytase plot, and for corn phytase are respectively 0·65, 0·67, and 0·59.

IV. Discussion

The ability of the bacterium to synthesize phytase in the absence of *myo*-inositol hexaphosphate implies that in this organism the enzyme does not require induction by substrate.

(*a*) *Preparation*

The desalting of the preparation on Sephadex G15 resulted in an approximately threefold increase in specific activity. This increase probably results from the selective adsorption of non-active protein on the Sephadex gel.

A further loss of protein and a relatively large loss of activity occurred during chromatography on Whatman DE32 anion-exchange cellulose. The cause of this loss of activity was not investigated.

The 25-fold increase in specific activity observed in the present work is less than the 1835-fold increase obtained by Nagai and Funahashi (1962) for wheat-bran phytase, but the final specific activity for *Pseudomonas* phytase is greater than that reported by these authors for wheat-bran phytase.

(*b*) *Properties of Partially Purified* Pseudomonas *Phytase*

(i) *pH Optimum*

The pH optimum characterizes the enzyme as an acid phosphomonoesterase. The value of 5·5 is in the range of those values reported for most plant phytases (Peers 1953; Nagai and Funahashi 1962; Preece and Grav 1962; Gibbins and Norris 1963; Chang 1967).

(ii) *Kinetics*

myo-Inositol hexaphosphate is hydrolysed by both wheat-bran phytase (Tomlinson and Ballou 1962) and *Pseudomonas* phytase (Cosgrove 1970) in a stepwise manner. In order to limit the kinetic study of the *Pseudomonas* phytase, as far as possible, to the hexaphosphate → pentaphosphate step the assays were carried out on a large scale and the maximum degree of hydrolysis thus limited to 5% of the total ester-phosphate present in the added substrate. Published kinetic studies of plant phytases have used assays in which the degree of hydrolysis far exceeded 5% and so the derived kinetic parameters in these studies apply to more than a single substrate.

The kinetics of the *Pseudomonas* phytase are complex and best discussed in relation to Figure 2(*b*). The particular form of this plot was chosen because it is claimed to accentuate any departure from linearity in the activity data (Hofstee 1959) while a comparison (Dowd and Riggs 1965) with the other two usual procedures has shown that it yields the most reliable estimates of K_m and $V_{max.}$ when Michaelis–Menten or steady-state conditions are obeyed. Had the data been plotted by the more usual method of Lineweaver and Burk (1934) the degree of curvature would have been reduced and a false impression of linearity created.

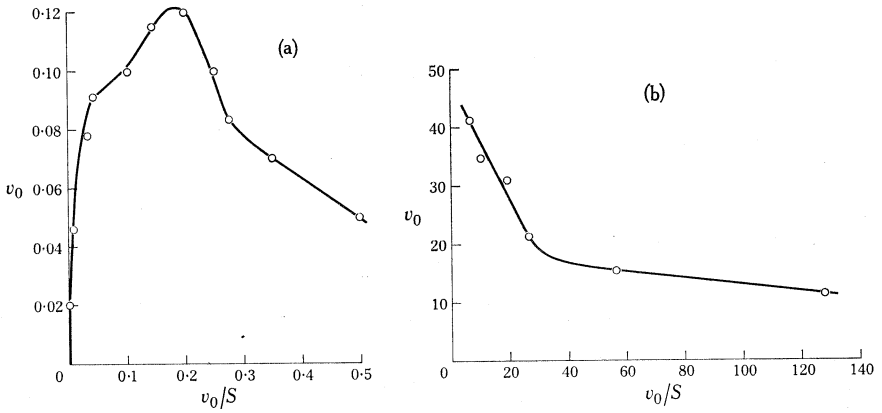

Fig. 5.—(*a*) A Woolf plot of the data, due to Gibbins and Norris (1963), for the activity of the phytase from *Phaseolus vulgaris* using *myo*-inositol hexaphosphate as substrate. The values used to compute the co-ordinates for this plot were estimated from the activity curve (Fig. 3) presented by Gibbins and Norris; v_0 is expressed as μmoles phytase converted per hour per millilitre of enzyme; S is the substrate concentration (mM). (*b*) A Woolf plot of the data, due to Chang (1967), for the activity of the phytase from *Zea mays* L. cv. Marcross, using *myo*-inositol hexaphosphate as substrate. The values used to compute the co-ordinates for this plot were taken from the text of Chang's paper; v_0 is expressed as micrograms inorganic phosphate liberated per hour per millilitre of enzyme; S is the substrate concentration (mM).

When the data of Gibbins and Norris (1963) for dwarf bean phytase [Fig. 5(*a*)] and of Chang (1967) for phytase from corn [Fig. 5(*b*)] were plotted using the Woolf procedure [cf. Fig. 2(*b*)] a similar departure from linearity was noted at low substrate concentrations.

Such plots [Figs. 2(*b*), 5(*a*), and 5(*b*)] need not imply the presence of a second enzyme, having a greater affinity for the substrate, since similar plots have been

obtained by Bechet and Yon (1964) for the hydrolysis of *p*-toluene sulphonyl-L-arginyl-methyl ester by purified trypsin.

At values of v_0/S below 0·0469 the kinetic behaviour of the enzyme is consistent with the model of partially non-competitive substrate inhibition discussed by Webb (1963).

If inhibition of *Pseudomonas* phytase by excess substrate is due to such a mechanism, a phosphorylated intermediate in a double-displacement mechanism would be required. There is some evidence that such intermediates exist in the reaction pathways of other acid phosphomonoesterases (Neuhaus and Byrne 1960; Greenberg and Nachmansohn 1965; Hou, Cleland, and Anderson 1966).

The failure, however, of Hou, Cleland, and Anderson (1966) and of Greenberg and Nachmansohn (1965) to isolate phosphorylated amino acids from hydrolyses of appropriately treated acid phosphomonoesterases suggests that if a phosphoryl–enzyme intermediate is formed it must be less stable than that of *Escherichia coli* alkaline phosphatase. Combination of a molecule of *myo*-inositol hexaphosphate with such an unstable intermediate in a *Pseudomonas* phytase hydrolysis could be expected to result in only a slight reduction in activity, yielding a curve of the type in Figure 2(*a*).

If Michaelis–Menten or steady-state conditions are assumed to apply for *Pseudomonas* phytase at values of v_0/S between 0·0082 and 0·0469 [Fig. 2(*b*)], then the computed K_m is $1 \cdot 63 \times 10^{-5}$M. This assumption that Michaelis–Menten or steady-state conditions are obeyed is supported by the linearity, over the same range of substrate concentrations, of the Woolf plots of the data for the activity of *Pseudomonas* phytase in the presence of magnesium ions [Fig. 2(*d*)].

The value for K_m lies outside the range of substrate concentrations used to compute it, but when it is applied to the activity curve [Fig. 2(*a*)] it yields a value of v_0 which is very nearly half the maximum velocity. It is therefore a realistic value for the K_m and could be used to characterize the enzyme. In this respect it is about one-quarter that obtained for wheat-bran phytase by Nagai and Funahashi (1962).

The apparent competitive inhibition of *Pseudomonas* phytase by magnesium ions requires more study. Comparison of Figure 2(*d*) with the figures used by Webb (1963, pp. 166–173) to illustrate specific types of inhibition, suggests that the inhibition of *Pseudomonas* phytase by magnesium ions is due to competition between magnesium and some cation essential for the binding of the substrate.

(iii) *Substrate Specificity*

Pseudomonas phytase differs from wheat-bran phytase (Nagai and Funahashi 1962) and resembles *B. subtilis* phytase (Powar and Jagannathan 1967) in that it appears to lack activity towards inorganic pyrophosphate, β-glycerophosphate, ADC, and AMP. Also, it has some activity toward *p*-nitrophenyl phosphate. These results may in part reflect the low substrate concentration used in the assays (0·36 mM). This concentration is lower than that conventionally used for these substrates, but use of a higher substrate concentration would have involved using as a reference an assay in which the *Pseudomonas* phytase was inhibited with respect to *myo*-inositol hexaphosphate as substrate.

The relative rates of hydrolysis of the inositol hexaphosphates (Table 2), being functions of both K_m and $V_{max.}$, cannot provide unequivocal evidence concerning

the binding of the substrates by the enzyme. They can, however, be used to help define those elements of structure in the substrate which are important for a maximum initial rate of hydrolysis under the conditions of assay. These structural features are used in the following paper (Irving and Cosgrove 1971) to help to derive a possible model of the active centre of *Pseudomonas* phytase.

The inhibition of the hydrolysis of *myo*-inositol hexaphosphate by the addition of an equimolar quantity of the D-*chiro*- or the *scyllo*- isomer needs more study, but the results suggest that all the isomers are hydrolysed at a single type of active centre on a single enzyme.

(iv) *Effect of Cation-complexing Agents*

Nagai and Funahashi (1962) found that 1 mM oxalate and 1 mM citrate were without effect on the activity of partially purified wheat-bran phytase. They found also that 1 mM tartrate was inhibiting while 1 mM EDTA was slightly activating. Peers (1953) on the other hand found that both 10 mM oxalate and 10 mM citrate activated a crude extract of wheat-bran phytase. The effects of these reagents are obviously complex.

The results with *Pseudomonas* phytase differ from those of Nagai and Funahashi (1962) in that 1 mM EDTA inhibits the enzyme. *Bacillus subtilis* phytase is also inhibited by EDTA (Powar and Jagannathan 1967).

(v) *Effect of Fluoride Ion*

The results [Section III(*b*)(v); Fig. 4] show that *Pseudomonas* phytase is apparently inhibited to a greater extent by fluoride ion than any of the plant phytases about which published data is available. A direct comparison is not possible due to differences in the assay conditions between this and the other studies.

If the difference in the slopes of the plot for *Pseudomonas* phytase and the plots for the plant phytases (Fig. 4) is due to an inherent difference between the two classes of enzymes, then the values of the slopes suggest that the inhibition of *Pseudomonas* phytase involves combination of three fluoride ions with a single active centre while fluoride inhibition of plant phytases involves combination of a single fluoride ion at each active centre.

V. References

Bechet, J. J., and Yon, J. (1964).—*Biochim. biophys. Acta* **89**, 117.

Chang, C. W. (1967).—*Cereal Chem.* **44**, 129.

Cosgrove, D. J. (1969).—*Ann. N.Y. Acad. Sci.* **165**, 677.

Cosgrove, D. J. (1970).—*Aust. J. biol. Sci.* **23**, 1207.

Cosgrove, D. J., Irving, G. C. J., and Bromfield, S. M. (1970).—*Aust. J. biol. Sci.* **23**, 339.

Desjobert, A., and Fleurent, P. (1954).—*Bull. Soc. Chim. biol.* **36**, 475.

Dowd, J. E., and Riggs, D. S. (1965).—*J. biol. Chem.* **240**, 863.

Gibbins, L. N., and Norris, F. W. (1963).—*Biochem. J.* **86**, 67.

Greaves, M. P., Anderson, G., and Webley, D. M. (1967).—*Biochim. biophys. Acta* **132**, 412.

Greenberg, H., and Nachmansohn, D. (1965).—*J. biol. Chem.* **240**, 1639.

Haldane, J. B. S., and Stern, K. G. (1932).—In "Allgemeine Chemie der Enzyme". p. 119. (Steinkoff Verlag: Dresden and Leipzig.)

Hofstee, B. H. J. (1959).—*Nature, Lond.* **184**, 1296.

HOU, R. Y., CLELAND, W. W., and ANDERSON, L. (1966).—*Biochemistry, N.Y.* **5**, 799.
HUNTER, A., and DOWNS, C. E. (1945).—*J. biol. Chem.* **157**, 427.
IRVING, G. C. J., and COSGROVE, D. J. (1970).—*Analyt. Biochem.* **36**, 381.
IRVING, G. C. J., and COSGROVE, D. J. (1971).—*Aust. J.biol. Sci.* **24**, 559.
LINEWEAVER, H., and BURK, D. (1934).—*J. Am. chem. Soc.* **56**, 658.
LONDON, M., MCHUGH, R., and HUDSON, P. B. (1958).—*Archs Biochem. Biophys.* **73**, 72.
LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951).—*J. biol. Chem.* **193**, 265.
NAGAI, Y., and FUNAHASHI, S. (1962).—*Agric. biol. Chem.* **26**, 794.
NEUHAUS, F. C., and BYRNE, W. L. (1960).—*J. biol. Chem.* **235**, 2019.
PEERS, F. G. (1953).—*Biochem. J.* **53**, 102.
POWAR, V. K., and JAGANNATHAN, V. (1967).—*Indian J. Biochem.* **4**, 184.
PREECE, I. A., and GRAV, H. J. (1962).—*Inst. Brew.* **68**, 66.
SLOANE-STANLEY, G. H. (1961).—In "Biochemists' Handbook". (Eds. C. Long, E. J. King, and W. M. Sperry.) (E. & F. N. Spon: London.)
TOMLINSON, R. V., and BALLOU, C. E. (1962).—*Biochemistry, N.Y.* **1**, 166.
WEBB, J. L. (1963).—"Enzyme and Metabolic Inhibitors." 1st Edn. pp. 118–30. (Academic Press: New York and London.)